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Intercellular Pectic Protuberances in *Asplenium*: New Data on their Composition and Origin

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• *Background and Aims* Projections of cell wall material into the intercellular spaces between parenchymatic cells have been observed since the mid-19th century. Histochemical staining suggested that these intercellular protuberances are probably pectic in nature, but uncertainties about their origin, composition and biological function(s) have remained.
• *Methods* Using electron and light microscopy, including immunohistochemical methods, the structure and the presence of some major cell wall macromolecules in the intercellular pectic protuberances (IPPs) of the cortical parenchyma have been studied in a specimen of the *Asplenium aethiopicum* complex.
• *Key Results* IPPs contained pectic homogalacturonan, but no evidence for pectic rhamnogalacturonan-I or xylogalacturonan epitopes was obtained. Arabinogalactan-proteins and xylan were not detected in cell walls, middle lamellae or IPPs of the cortical parenchyma, whereas xyloglucan was only found in its cell walls. Extensin (hydroxyproline-rich glycoproteins) LM1 and JIM11 and JIM20 epitopes were detected specifically in IPPs but not in their adjacent cell walls or middle lamellae.
• *Conclusions* It is postulated that IPPs do not originate exclusively from the middle lamellae because extensins were only found in IPPs and not in surrounding cell walls, intercellular space linings or middle lamellae, and because IPPs and their adjacent cell walls are discontinuous.

Key words: Pteridophyta, *Asplenium*, immunohistochemistry, pectic polysaccharides, extensin, cell wall, fern.

INTRODUCTION

Aspleniaceae, with over 720 terrestrial, lithophytic or epiphytic species distributed throughout the world, is one of the largest families within the Filicopsida. During a comparative anatomical study of *Asplenium* species, projections on cell wall surfaces into intercellular spaces in the cortical parenchyma of the petioles were noted. According to Kisser (1928), De Vriese and Harting were the first to report such excrescences in 1853. Subsequently, similar structures were found in the intercellular spaces of seeds, leaves, stems and roots of many monocotyledons, dicotyledons, ferns and fern allies (for a review see Potgieter and van Wyk, 1992). These have been referred to as intercellular wall thickenings (Luerssen, 1873), pectic strands (Carr and Carr, 1975; Carr *et al.*, 1980*a*, *b*), pectic filaments (Carr and Carr, 1975; Potgieter and van Wyk, 1992), pectic warts (Kisser, 1928; Carlquist, 1956, 1957), scala (Potgieter and van Wyk, 1992), pectic projections (Davies and Lewis, 1981; Veys *et al.*, 1999, 2000, 2002), microprojections (Rolleri, 1993), beads (Jeffree and Yeoman, 1983; Barnett and Weatherhead, 1988), bead-like projections (Miller and Barnett, 1993), papilla-like structures (Suske and Acker, 1989), protuberances (Donaldson and Singh, 1984), intercellular protuberances (Butterfield *et al.*, 1981; Machado *et al.*, 2000), and intercellular pectic protuberances (IPP) (Potgieter and van Wyk, 1992; Machado and Sajo, 1996; Rolleri, 2002; Mengascini, 2002; Prada and Rolleri, 2005; Rolleri and Prada, 2006). Besides *Asplenium*, protuberances in ferns and fern allies have been studied in *Angiopteris* (Carr and Carr, 1975; Rolleri, 2002; Mengascini, 2002), *Pteridium* (Carr and Carr, 1975), *Pteris* (Schenck, 1886), *Blechnum* (Schenck, 1886), *Christensenia* (Rolleri, 1993), *Isoetes* (Prada and Rolleri, 2005), *Equisetum* (Vidal, 1896), *Azolla* (Veys *et al.*, 1999, 2000, 2002) and *Marattia* (Lavalle, 2003).

Infraspecific variability in the occurrence, form and distribution of IPPs has been reported for some Hawaiian Asteraceae (Carlquist, 1957), some southern African Icacinaceae (Potgieter and van Wyk, 1992), some *Isoetes* species (Prada and Rolleri, 2005), and for some *Blechnum* species (Rolleri and Prada, 2006). Nevertheless, Potgieter and van Wyk (1992) emphasized the need for more anatomical studied to asses the variability at lower taxonomic levels.

Based on tests using different dyes, such as methylene blue and naphthalene blue, the pectic nature of the protuberances was first postulated by Mangin (1892, 1893). Since then many authors identified pectin as the main constituent of IPPs (Table 1). However, 'pectins' constitute a family of polysaccharides rich in galacturonic acid (GalA), and subdivided into three main classes: homogalacturonan (HG), rhamnogalacturonan-I (RGI) and

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TABLE 1. *Chronological review of studies including test(s) to identify putative chemical component(s) and origin of IPPs*

Reference	Taxon	Dye(s) or histochemical test(s)	Putative chemical composition	Postulated origin
Mangin (1892, 1893)	*Blechnum*, *Pteris*, *Equisetum*, *Vitis*, *Yucca*	Methylene blue, Naphtalene blue, etc.	Pectin	–
Carré and Horne (1927)	*Malus*	Ruthenium red	Pectin	Secretion
Kisser (1928)	*Aconitum*, *Helleborus*, *Saxifraga*, *Daphne*, *Eriobotrya*, *Fatsia*, *Erica*, *Yucca*, *Dieffenbachia, Asplenium*	Ruthenium red	Pectin	Secretion
Carlquist (1956)	*Fitchia*, *Wyethia*	Ruthenium red	Pectin	Secretion
Carlquist (1957)	*Argyroxiphium*, *Wilkesia*	Ruthenium red	Pectin	Secretion
Carr and Carr (1975)	*Eucalyptus*	–	Pectin	Rearranged middle lamella
Carr and Carr (1975)	*Angiopteris, Pteridium*	–	Pectin	Possibly secretion
Carr *et al.* (1980*a*)	*Vicia, Scilla, Iresin, Helianthus*	Pectinase	Pectin	Rearranged middle lamella
Butterfield *et al.* (1981)	*Cocos*	Ruthenium red	Pectin	Secretion
Davies and Lewis (1981)	*Daucus*	Ruthenium red Sudan IV	Pectin Neutral lipids	–
Jeffree and Yeoman (1983)	*Lycopersicon*	Ruthenium red and hydroxylamine/ ferric chloride	Pectin	Secretion
Donaldson and Singh (1984)	*Pinus*	Toluidin blue	Pectin	–
Potgieter and van Wyk (1992)	*Apodytes*, *Cassinopsis*, *Pyrenacantha*	Ruthenium red and hydroxylamine/ ferric chloride	Pectin	Rearranged middle lamella
Miller and Barnett (1993)	*Picea*	Ruthenium red and hydroxylamine/ ferric chloride Nile blue Coomassie Brilliant Blue R Aniline blue Calcofluor	Pectin Fatty acids Protein Callose Cellulose	Secretion
Rolleri (1993)	*Christensenia*	Ruthenium red	Pectin	–
Machado and Sajo (1996)	*Xyris*	Ruthenium red	Pectin	–
Veys *et al.* (1999)	*Azolla*	Ruthenium red Alcian blue/Toluidine blue Aniline blue, Resorcine blue Coomassie Brilliant Blue R	Pectin Acidic polysaccharides Callose Proteins	Secretion
Machado *et al.* (2000)	*Paepalanthus*	Ruthenium red Sudan IV/Sudan black	Pectin Lipids	Secretion
Tiné *et al.* (2000)	*Hymenaea*	Iodine PAS reagent	Xyloglucan Carbohydrates	Rearranged middle lamella
Rolleri (2002)	*Angiopteris*	Ruthenium red	Pectin	–
Veys *et al.* (2002)	*Azolla*	Ruthenium red Coomassie Brilliant Blue R	Pectin Proteins	Secretion
Prada and Rolleri (2005)	*Isoetes*	Toluidine blue O	Pectin	–
Leroux (2005)	*Asplenium anisophyllum-complex*	Ruthenium red/pectinase	Pectin	Possibly secretion

–, Authors did not provide data.

rhamnogalacturonan-II (RGII) (Willats *et al.*, 2006). HG is a linear polymer consisting of 1,4-linked α-D-GalA that can carry varying patterns and densities of methylesterification. RGI consists of the repeating disaccharide [→4)-α-D-GalA-(1→2)-α-L-rhamnose-(1→] on which a variety of different glycan chains (principally arabinan and galactan) can be attached to the rhamnose residues. RGII has a backbone of HG rather than RG, with complex side chains attached to the GalA residues (Willats *et al.*, 2006). Using chemical stains, other constituents of the primary cell wall were also detected in IPPs: cellulose in *Picea* (Miller and Barnett, 1993), xyloglucan in *Hymenaea* (Tiné *et al.*, 2000), proteins and callose in *Azolla* (Veys *et al.*, 1999).

The interest in cell walls has increased over the last decennia, and aspects of pteridophyte and other non-angiosperm plants were studied by Popper and Fry (2003), Popper *et al.* (2004), Matsunaga *et al.* (2004), Popper and Fry (2004), Carafa *et al.* (2006), Popper (2006) and Johnson and Renzaglia (2007). However, only Carafa *et al.* (2006) and Johnson and Renzaglia (2007) used monoclonal antibodies to investigate cell wall composition in pteridophytes.

Apart from the composition of IPPs, many uncertainties concerning their development remain. Are they formed from materials of the middle lamella during the formation of intercellular spaces, or do they consist of material secreted onto cell walls after the formation of intercellular spaces? The pectic nature of IPPs led most researchers (e.g. Carr and Carr, 1975; Carr *et al.*, 1980*a*; Potgieter and van Wyk, 1992; Tiné *et al.*, 2000) to conclude that IPPs are most probably formed during cell separation.

They suggest that when cells are pulled apart during parenchyma expansion, the pectin of the middle lamella becomes stretched and forms strands between adjacent cells. Protuberances then form after the rupture of these strands as parenchyma development proceeds. In contrast to this view, other authors stress the possibility that the pectic filaments observed in ferns (Potgieter and van, Wyk 1992; Carr and Carr, 1975) and other plant groups (Machado *et al.*, 2000; Butterfield *et al.*, 1981; Davies and Lewis, 1981) may be derived from new materials laid down after intercellular space formation. According to Veys *et al.* (2002) protuberances appear both under stress conditions, such as wounding (Davies and Lewis, 1981) and grafting (e.g. Donaldson and Singh, 1983; Miller and Barnett, 1993), and unstressed conditions (e.g. Carlquist, 1956; Potgieter and van Wyk, 1992).

Mostly based on their pectic nature, several possible functions of the intercellular protuberances have been suggested, such as cell wall hydration, storage, cell adhesion, defence and apoplastic transport (Potgieter and van Wyk, 1992), but none has been confirmed.

Although many authors have described morphological aspects of IPPs, many uncertainties concerning their origin and composition remain. In this paper, the intercellular protuberances in *Asplenium* are described. Using immunohistochemistry and electron microscopy, new data on their origin and composition are presented.

MATERIALS AND METHODS

Plant material

Sporophyte material was sampled from plants cultivated at Ghent University Botanical Garden. After examining *Asplenium aethiopicum*, *A. anisophyllum*, *A. balense*, *A. decompositum*, *A. javorkeanum, A. nitens, A. normale*, *A. onopteris A. protensum*, *A. scolopendrium*, *A. simii*, *A. smedsii*, *A. splendens*, *A. trichomanes* and *A. uhligii*, one specimen (Viane 8539; pers. Herb. R. Viane, GENT) was selected from the *A. aethiopicum* complex for a more detailed analysis. Sections of both juvenile uncurling croziers and mature leaves were made.

Light microscopy

Tissues were embedded using the Technovit 7100 embedding kit (Heraeaus Kulzer, Wehrheim, Germany). Small pieces of plant material (root, rhizome, petiole and lamina), measuring approx. 4 mm on all sides, were excised using razor blades, and fixed in FAA (90 % ethanol 50 %, 5 % acetic acid and 5 % commercial formalin). Dehydration was performed using 30 %, 50 %, 70 %, 85 % and 94 % ethanol. After the last alcohol step, the tissue was infiltrated with hydroxyethylmethacrylate-based resin. Since the samples used in this study are larger then those mentioned in Beeckman and Viane (2000), longer infiltration steps were required. Further treatment was according to Beeckman and Viane (2000), using the one-step embedding method of De Smet *et al.* (2004). Some samples were embedded in custom-made Teflon moulds. Transverse sections of 5 μm were cut with a microtome (Minot 1212, Leitz, Wetzlar, Germany), dried on object glasses, and stained with an aqueous 0·05 % (w/v) solution of toluidine blue O (Merck, Darmstadt, Germany, C.I. No. 52040) in 0·1 % $Na_2B_4O_7$. A histochemical test with aqueous 1 % (w/v) ruthenium red (Sigma, St Louis, MO, USA) was performed to indicate the presence of pectic material. Hand sections were treated with safranine O (Johansen, 1940) and phloroglucinol (Johansen, 1940) to test for lignin, and aniline blue (0·01 % in 0·1 M phosphate buffer, pH 9) to demonstrate callose. Micrographs were taken using a Canon EOS D10 mounted on an Olympus BH2 microscope.

Scanning electron microscopy

Material was washed twice with 70 % ethanol for 5 min and then placed in a mixture (1 : 1) of 70 % ethanol and DMM (dimethoxymethane) for 20 min. Subsequently, the material was transferred to 100 % DMM for 20 min, before it was CO_2 critical point dried using a CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). The dried samples were mounted on aluminium stubs using Leit-C and coated with gold with a SPI-ModuleTM Sputter Coater (SPI Supplies, West-Chester, PA, USA). Images were obtained on a Jeol JSM-6360 (Jeol, Tokyo) at the Laboratory of Plant Systematics (K.U. Leuven).

Transmission electron microscopy

Petiole cortex tissue was cut into blocks measuring approx. 2 mm on all sides. These were fixed with 2 % formaldehyde and 2·5 % glutaraldehyde in a cacodylate buffer 0·1 M pH 7·4 for 24 h at 4 °C, washed in the same buffer for 8 h, post-fixed with 4 % osmiumtetroxide and dehydrated in a step gradient of ethanol at room temperature. The samples were transferred to 100 % alcohol/Spurr's resin (1 : 1) at 4 °C overnight, brought to 100 % alcohol/Spurr's resin (1 : 2) for 8 h (4 °C), and transferred to 100 % Spurr's resin and left overnight at 4 °C. Polymerization was performed at 70 °C for 16 h. Sections (70 nm thick) were made using a Reichert Ultracut S Ultramicrotome. Formvar-coated single slot copper grids were used. Sections were post-stained with a Leica EM stain for 30 min in uranyl acetate at 40 °C and 10 min in lead citrate stain at 20 °C. The grids were examined with a JEM 1010 Jeol electron microscope equipped with imaging plates which were scanned digitally (Ditabis, Pforzheim, Germany).

Immunohistochemistry

The molecular composition of cell walls and IPPs was determined using indirect immunofluorescence with a range of monoclonal antibodies directed against cell wall polysaccharides/glycoproteins. These included: anti-HG monoclonal antibodies JIM5, JIM7 and LM7 (Clausen *et al.*, 2003) and PAM1 (Manfield *et al.*, 2005); anti-xylogalacturonan LM8 (Willats *et al.*, 2004); anti-galactan LM5 (Jones *et al.*, 1997), anti-arabinan LM6 (Willats

et al., 1998); anti-arabinogalactan-protein antibodies LM2 (Smallwood *et al.*, 1996), JIM4 (Knox *et al.*, 1989), JIM13, JIM14 (Knox *et al.*, 1991) and MAC207 (Pennell *et al.*, 1989); anti-extensin antibodies LM1 (Smallwood *et al.*, 1995), JIM11, JIM12, JIM19 and JIM20 (Smallwood *et al.*, 1994); anti-xylan antibody LM11 (McCartney *et al.*, 2005) and a new anti-xyloglucan monoclonal antibody recognizing the XXXG motif of xyloglucans (unpubl. res.).

Petioles stored in 70 % ethanol were sectioned by hand and immediately placed in a fixative of 4 % paraformaldehyde in 50 mM Pipes, 5 mM $MgSO_4$ and 5 mM EGTA. Following overnight fixation, sections were washed in PBS and then incubated for 1 h in primary antibody diluted in 5 % milk protein in PBS. All rat monoclonal antibodies were used as a 5-fold dilution. Sections were washed in PBS prior to incubation for 1 h in secondary antibody. The secondary antibody was anti-rat IgC coupled to flourescein isothiocyanate (FITC) (Sigma). Soluble scFV of PAM1 was incubated with sections at 20 μg mL^{-1} followed by, a 100-fold dilution of mouse anti-HIS as secondary antibody. Next, a 50-fold dilution of anti-mouse/FITC was used as tertiary antibody. After washing in PBS, all sections were mounted in anti-fade agent (Citifluor, Agar Scientific) and examined using a microscope equipped with epifluorescence irradiation and DIC optics (Olympus BX-61). Images were captured with a Hamamatsu ORCA285 camera and prepared with Improvision Volocity software. Cellulose was stained with Calcofluor White M2R fluorochrome (fluorescent brightener 28; Sigma; 0·25 μg mL^{-1} in dH_2O). In some cases plant material embedded in resin, as described above, was used for immunohistochemistry.

Peroxidase activity

In situ peroxidase activity was detected in fresh hand-cut sections of cortical parenchyma following pretreatment in 5 mg ml^{-1} 3,3′-diaminobenzidine (DAB)-HCL, pH 3·8, re-buffered to pH 5·8 immediately before use. Subsequently 1 mM H_2O_2 was added and incubation was performed at room temperature for 5 min.

RESULTS

IPPs were found in all the *Asplenium* species studied. A cross-section of the petiole of a mature *Asplenium* leaf typically shows two vascular bundles (Fig. 1A), which fuse towards the rachis. The outermost layer or epidermis is followed internally by a hypodermis consisting of strongly sclerified cells. The largest part of the cortex consists of parenchyma with intercellular spaces (Fig. 1A), in which IPPs are visible at magnifications above ×200 (Fig. 1B). No regular arrangement of pectic strands connecting adjacent cells was seen.

Sections through superimposed zones of juvenile petioles bearing a crozier show that in the upper zone with undifferentiated metaxylem vessels, the minute intercellular spaces in the parenchyma lack IPPs. In sub-mature tissues IPPs are rare but intercellular space corners are frequently filled with IPP material. Mature petiole bases have large intercellular

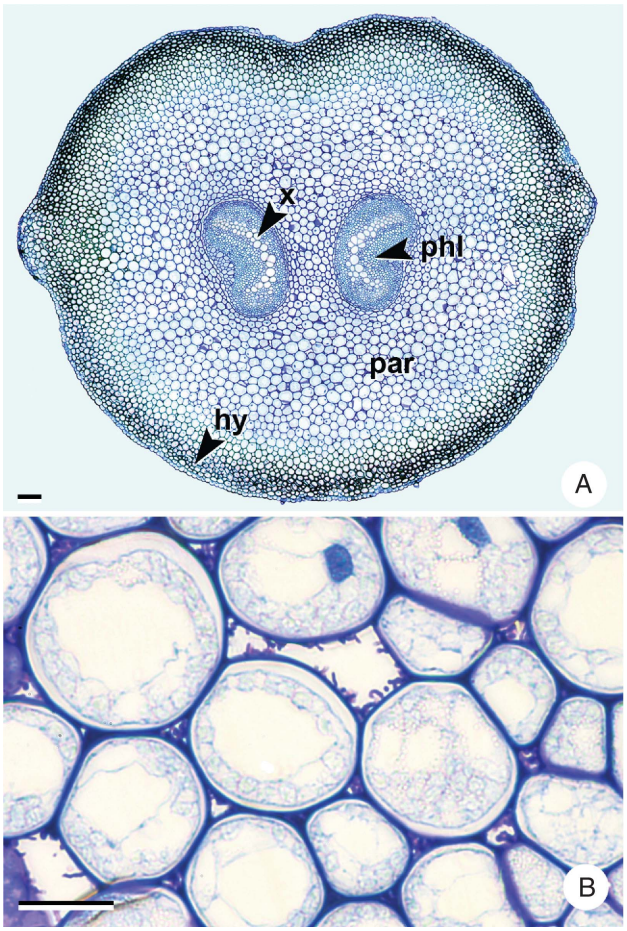

FIG. 1. Transverse sections of the petiole of a specimen of the *Asplenium aethiopicum* complex (RV 8539). (A) Light micrograph showing two vascular bundles surrounded by cortical parenchyma. (B) Detail of cortical parenchyma with numerous protuberances lining the intercellular spaces. x, Xylem; phl, phloem; par, cortical parenchyma; hy, hypodermis. Scale bars: (A) = 125 μm; (B) = 30 μm.

spaces with numerous IPPs. All the mature tissues studied containing intercellular spaces, i.e. the petiolar cortical parenchyma and the laminal mesophyll, possess IPPs. However, relatively few protuberances are present in the mesophyll. Rhizomes have only few and small intercellular spaces that contain very thin filamentous IPPs. Since intercellular spaces are absent in *Asplenium* roots, no cell wall protuberances were observed in this organ.

Ruthenium red showed a positive reaction with the protuberances, but tests for lignin and for callose were negative. The cellulose-binding fluorophor calcofluor did not label IPPs but clearly bound to neighbouring cell walls (Fig. 3C). After performing a peroxidase activity test by adding DAB and H_2O_2 to fresh hand-cut sections, all IPPs in the cortical parenchyma were stained.

Scanning electron micrographs of the cortical parenchyma show protuberances of irregular size and shape, without any regular arrangement or strands connecting adjacent cells (Fig. 2A). Warts and nodulated filaments are smooth and appear to be firmly attached to the surface of the cell wall (Fig. 2B).

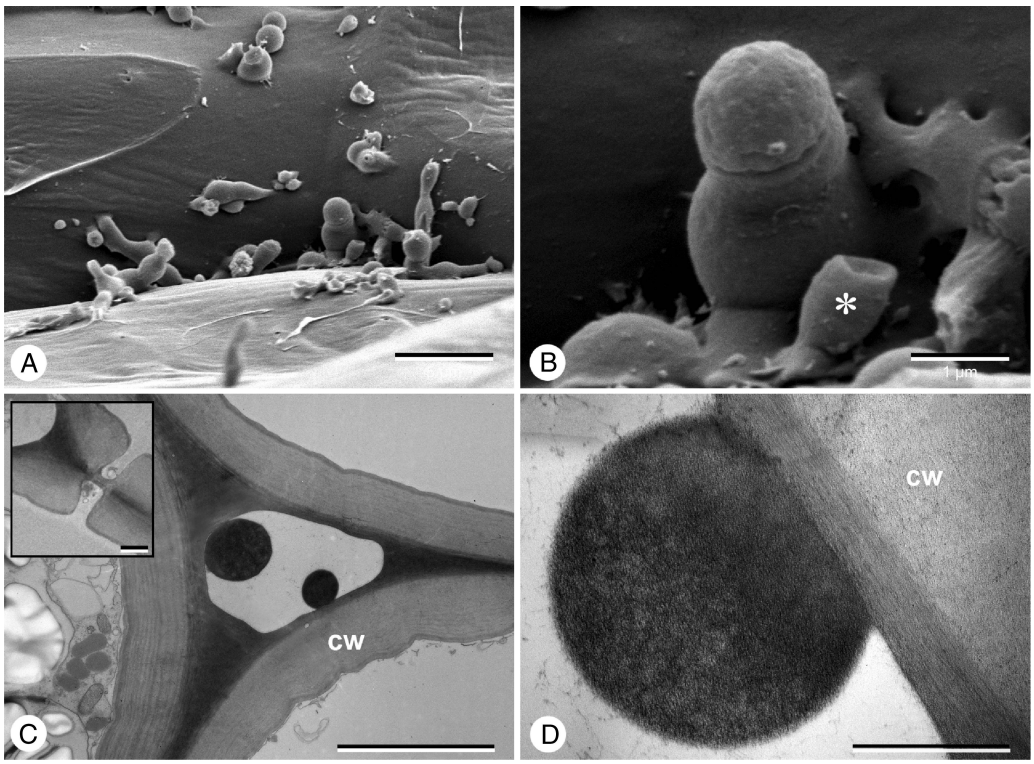

FIG. 2. IPPs in cortical parenchyma of a specimen of the *Asplenium aethiopicum* complex (RV 8539). (A) SEM micrograph showing protuberances on cell surfaces in the intercellular space. (B) Detail of a nodulated filament; note some slightly collapsed protuberances due to solvent extraction during processing (asterisk). (C) TEM micrograph of an intercellular space with two wart-like IPPs showing discontinuity in electron-density with the adjacent cell walls (cw). Inset: TEM micrograph showing a simple pit. (D) TEM detail of a protuberance showing an internal electron-dense fibrillar network. Scale bars: (A) = 5 μm; (B) = 1 μm; (C) = 4 μm; inset in (C) = 1 μm; (D) = 0·6 μm.

Transmission electron microscopy shows that cell walls are clearly layered and possess simple pits (Fig. 2C, inset). The shapes of IPPs observed with transmission electron microscopy agree with those seen by scanning electron microscopy; their internal structure seems rather homogenous and shows no variation between samples. At higher magnifications an electron-dense fibrillar network is visible within IPPs. The adjacent cell wall shows a more electron-dense outer zone or lining, but there is no continuity between the protuberances and the cell wall (Fig. 2D).

The monoclonal antibodies JIM5 and JIM7, which bind to a range of partially methyl-esterified homogalacturonan domains, clearly label the region of the cell wall lining the intercellular spaces between the cortical parenchyma cells. At higher magnifications (Fig. 3A) the abundance of the JIM5 epitope in the middle lamellae, the intercellular space linings and IPPs is evident. The blockwise de-esterified homogalacturonan epitope recognized by PAM1 binds both to the intercellular space linings and to IPPs (Fig. 3D, E). It is of interest that LM5, LM6, LM7 and LM8, monoclonal antibodies that bind to other pectic polymers, did not bind to any structure or part of the cortical parenchyma of *Asplenium*. However, the LM6 probe for arabinan bound to all cell walls of the vascular bundle including the endodermis (Fig. 3G).

For the non-pectic polymers it was found that none of the anti-arabinogalactan-protein antibodies LM2, JIM4, JIM13, JIM14 and MAC207 bound to cortical parenchyma of mature leaves, and that the anti-extensin monoclonal antibodies LM1, JIM11 and JIM20 bound exclusively to IPPs but not to associated cell walls or middle lamellae. The distribution of the LM1 extensin epitope is shown in Fig. 3H and I. The monoclonal antibody to the XXXG motif of xyloglucan did not label IPPs, although this antibody did bind to the parenchyma cell walls (Fig. 3F). The simple pits, observed by transmission electron microscopy (Fig. 2C, inset), were also visualized by this probe. Anti-xylan LM11 bound to the secondary cell walls of tracheids in the vascular tissue but not to cortical parenchyma cell walls, IPPs or middle lamellae. An overview of the immunohistochemical observations made is shown in Table 2.

DISCUSSION

Intercellular protuberances occur in tissues of various ferns and fern allies, gymnosperms and angiosperms (Potgieter and van Wyk, 1992). Because detailed information on protuberances is restricted to relatively few taxa, we emphasize that protuberances with a similar morphology are not necessarily ontogenetically, chemically or anatomically identical.

The observations of Luerssen (1875) and Kisser (1928) were confirmed in the present study but big stalked spherical bodies were found in not only *A. scolopendrium* but in

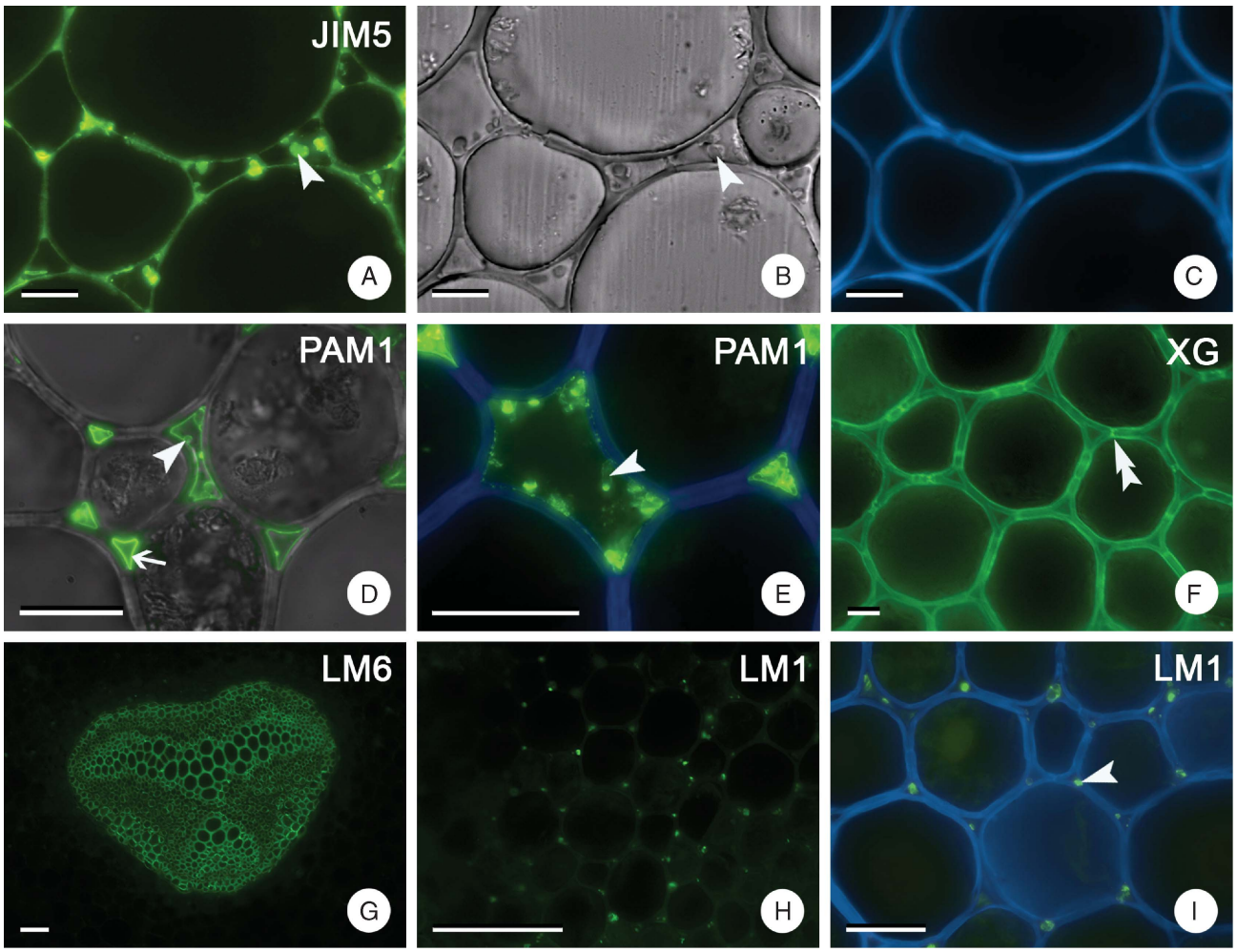

FIG. 3. Indirect immunofluorescence labelling of cell wall polymers in transverse hand-cut and resin-embedded petiole sections of a specimen of the *Asplenium aethiopicum* complex (RV 8539). (A) Intercellular spaces with IPPs in the cortical parenchyma (resin-embedded): immunolabelling of HG with JIM5 showing labelled middle lamellae and IPPs. (B) Same section as in (A): differential interference contrast (DIC). (C) Same section as in (A): reaction of cellulose-binding fluorophor calcofluor: note IPPs are not stained. (D) Immunolabelling of unesterified HG with PAM1; bright field/fluorescence micrograph showing binding to IPPs (hand-cut section). (E) Detail of cortical parenchyma labelled with PAM1 and stained with calcofluor (hand-cut section). (F) Immunolabelling of xyloglucan: note IPPs are not stained (hand-cut section). (G) Immunolabelling of RG-I related (1 → 5)-α-L-arabinan with LM6: note only the vascular bundle is labelled (hand-cut section). (H) Immunolabelling of extensin with LM1: only IPPs are labelled (hand-cut section). (I) Detail of cortical parenchyma labelled with LM1 and stained with calcofluor (hand-cut section). Arrowheads and arrows indicate IPPs and linings of intercellular spaces, double arrowheads indicate simple pits. Scale bars: (A–C, F) = 12 μm; (D, E, H, I) = 30 μm; (G) = 100 μm.

all the *Asplenium* species studied. IPPs in *Asplenium* are more numerous in the petiole than in the mesophyll. Those in the rhizome resemble the filaments described in the rhizome parenchyma of *Pteridium esculentum* (Carr and Carr, 1975). However, intercellular spaces in the rhizome of *Asplenium* are smaller and protuberances rare.

In contrast to *Cassinopsis ilicifolia* (Potgieter and van Wyk, 1992), transmission electron microscopical observations of the samples used in the present study showed dissimilarities in electron density between IPPs and their adjacent cell walls and middle lamellae. Remarkably, the primary parenchyma cell walls in this specimen are composed of several layers. This phenomenon has been observed in primary cell walls when they consist of a series of layers in which the orientation of the microfibrils changes by a constant and usually small angle from one layer to the next (Brett and Waldron, 1996).

The protuberances in *Asplenium* show a positive reaction with ruthenium red, supporting their pectic composition. However, results based on reactions with ruthenium red should be interpreted with care because this stain may not be specific enough for pectic compounds (Krishnamurthy, 1999).

The negative tests for lignin and callose seem to indicate the absence of these compounds in IPPs, associated cell walls and middle lamellae. Although cellulose was clearly detected in associated cell walls, it is not found in the protuberances.

As a literature study showed that pectin was believed to be the main substance of intercellular protuberances of many plants, the present immunohistochemical tests

TABLE 2. *Reactions in the cortical parenchyma of a specimen of the* Asplenium aethiopicum *complex (RV 8539) to labelling with monoclonal antibodies directed against cell wall polysaccharides, proteins and glycoproteins*

	Antibody	Epitope	Cortex cell walls	Intercellular space linings	Middle lamella	IPPs
Pectin						
Homogalacturonan (HG)	JIM 5	Partially methyl-esterified HG/unesterified HG	−	+	+	+
	JIM 7	Partially methyl-esterified HG	−	+	+	+
	LM7	Partially methyl-esterified HG	−	−	−	−
	PAM1	Unesterified HG	−	+	−	+
Rhamnogalacturonan I (RG-I)	LM5	(1→4)-β-galactan	−	−	−	−
	LM6	(1→5)-α-arabinan	−	−	−	−
Xylogalacturonan	LM8		−	−	−	−
Arabinogalactan-protein (AGP)	JIM 4	AGP glycan	−	−	−	−
	JIM 13	AGP glycan	−	−	−	−
	JIM 14	AGP glycan	−	−	−	−
	MAC207	AGP glycan	−	−	−	−
Extensin	LM1	Extensin	−	−	−	+
	JIM 11	Extensin	−	−	−	+
	JIM 19	Extensin	−	−	−	−
	JIM 20	Extensin	−	−	−	+
Xyloglucan	Name unpublished	XXXG motif	+	−	?	−
Xylan	LM11	Unsubstituted xylan/arabinoxylan	−	−	−	−

+, Binding; −, no binding; ?, binding has to be confirmed by immunogold microscopy.

mainly screened for pectins. The fact that monoclonal antibodies JIM5, JIM7 and PAM1 bind to cell walls and to IPPs indicates the abundance of a mixture of unesterified and methyl-esterified HG. It is of interest that LM7, which binds to a non-blockwise partially methyl-esterified epitope of HG and is restricted to corners of intercellular spaces in angiosperm parenchyma (Willats *et al.*, 2001), did not bind to any material in embedded or hand-cut sections of *Asplenium* cortical parenchyma. Similarly, LM8, an antibody binding to a xylogalacturonan epitope associated with cell detachment in a range of systems (Willats *et al.*, 2004), did not bind to *Asplenium* cortical parenchyma cells, nor did LM5 and LM6, probes for the possible side chains of pectic RGI: (1→4)-β-galactan and (1→5)-α-arabinan, respectively, which have often been correlated with stages of cell and/or tissue development (Ridley *et al.*, 2001).

Using a monoclonal antibody to the XXXG motif, xyloglucan was detected in cell walls of the cortical parenchyma, but not in IPPs. Consequently, IPPs in *Asplenium* do not have the same chemical nature as the xyloglucan containing protuberances in the storage cell walls in cotyledons of *Hymenaea courbaril* (Tiné *et al.*, 2000). Arabinogalactan-proteins were not detected in the cortical parenchyma cell walls or IPPs of the material used in the present study, even though the same extensive range of monoclonal antibodies that was used to demonstrate their presence in angiosperms (Showalter, 2001) had been applied.

Extensins were detected exclusively in IPPs, indicating that IPP material is distinct from adjacent primary cell walls and middle lamellae. Extensins are abundant constituents of the primary cell walls in mono- and dicotyledons (Smallwood *et al.*, 1995) where they are involved in cross-linking and strengthening processes. As IPPs were stained after performing a peroxidase activity test, extensins in IPPs of *Asplenium* could be cross-linked into IPP structures by this enzyme activity (Everdeen *et al.*, 1988; Brownleader *et al.*, 1993). In addition, extensins are cationic glycoproteins and therefore also have the potential to interact ionically with acidic pectins (Kieliszewski and Lamport, 1994). The distribution pattern of the PAM1 epitope indicates an abundance of unesterified pectin in the IPPs and they therefore may be structurally stabilized by an interaction with extensins.

Few articles report on localization patterns of peroxidases in fern cell walls. Ros *et al.* (2007) showed that structural motifs characteristic of eudicot *S*-peroxidases predate the radiation of tracheophytes, since they are found not only in peroxidases from ferns (*Ceratopteris*), lycopods and basal gymnosperms, but also in peroxidases of mosses and liverworts.

The majority of researchers hypothesize that IPPs are remnants of the middle lamellae, most probably formed during cell separation (e.g. Potgieter and van Wyk, 1992; Tiné *et al.*, 2000) when stretched middle lamellae strands snap and form filamentous or wart-like protuberances. However, their presence does not necessarily mean that they are derived from middle lamella components. Substances may be secreted at the site of future intercellular spaces, but prior to their development and expansion, and become incorporated in IPPs during intercellular space formation.

In *Asplenium*, the occasional observation of protuberances stretching completely across an intercellular space, i.e. from the outside of a wall of one parenchyma cell to the outside of another cell wall, could be consistent with the first hypothesis. However, extensins were only detected in IPPs and not in adjacent cell walls or middle lamellae, indicating a composition distinct from that of the middle lamellae. In addition, transmission electron microscope micrographs showed a clear discontinuity between the protuberances and the cell wall. Overall, these observations

strongly suggest that IPPs do not originate exclusively from the middle lamellae.

In summary, this is the first report on the composition of IPPs using monoclonal antibodies directed against cell wall molecules. Homogalacturonan and extensin hydroxyproline-rich glycoproteins in IPPs were identified. These results, in combination with transmission electron microscopical observations, suggest that IPPs do not have to originate exclusively from the middle lamellae because additional substances may be secreted during tissue development and become incorporated in IPPs during intercellular space formation. Further biochemical and ontogenetical research on the formation of intercellular spaces is needed to elucidate the composition and possible functions of IPPs in many vascular plants.

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